Functional Reconstitution of the Bovine Brain $GABA_A$ Receptor from Solubilized Components†

Susan M. J. Dunn,*,‡ Colleen R. Martin, Michael W. Agey, and Ryoko Miyazaki

Department of Physiology and Biophysics, The University of Iowa, Iowa City, Iowa 52242

Received August 9, 1988; Revised Manuscript Received October 27, 1988

ABSTRACT: The $GABA_A$/benzodiazepine receptor has been solubilized from membrane preparations of bovine cerebral cortex and has been reconstituted, in a functionally active form, into phospholipid vesicles. In preliminary experiments, the receptor was labeled with the photoactive benzodiazepine [^{3}H]flunitrazepam prior to solubilization. A peptide of apparent molecular weight 53 500 was specifically labeled by this method, and this was used as a marker for the receptor during the reconstitution procedures. The labeled protein was solubilized with approximately 40% efficiency by 1% β-octyl glucoside. Reconstitution was achieved by mixing the solubilized proteins with a 4:1 mixture of soybean asolectin and bovine brain phospholipids, followed by chromatography on Sephadex G-50-80 to remove detergent. The incorporation of the $GABA_A$ receptor into membrane vesicles has been verified by sucrose gradient centrifugation in which the [^{3}H]-flunitrazepam-labeled peptide comigrated with [^{14}C]phosphatidylcholine used as a lipid marker. Vesicles prepared without labeled markers retained the ability to bind both [^{3}H]flunitrazepam and the GABA analogue [^{3}H]muscimol. Furthermore, the binding parameters were very similar to those measured using native membrane preparations. A novel fluorescence technique has been used to measure chloride transport mediated by the $GABA_A$ receptor in reconstituted vesicles. Chloride influx was rapidly stimulated in the presence of micromolar concentrations of muscimol and was blocked by preincubation of the membranes with muscimol (desensitization). Flux was also blocked by pretreatment with the competitive $GABA_A$ receptor blocker bicuculline or with the noncompetitive $GABA_A$ receptor antagonist picrotoxin.

In recent years, considerable progress has been made in the study of the structural properties of the $GABA_A$ receptor. The receptor from mammalian brain has been purified by benzodiazepine affinity chromatography and has been shown to be formed by two subunits of apparent molecular weights 53 000 (α) and 57 000 (β) (Sigel et al., 1983; Sigel & Barnard, 1984; Schoch et al., 1984; Kuriyama & Taguchi, 1987). Recently, Schofield et al. (1987) reported the cloning and sequencing of the cDNAs encoding the two subunits. Coexpression of the in vitro generated subunit RNAs in *Xenopus* oocytes produced a functional γ-aminobutyric acid (GABA)[1] receptor whose channel properties were modulated with appropriate pharmacological specificity by benzodiazepines and barbiturates. These experiments confirm that the minimum requirement for $GABA_A$ receptor function is the presence of an α and a β subunit (Schofield et al., 1987).

The $GABA_A$ receptor has been extensively studied not only because of the importance of GABA as an inhibitory neurotransmitter in the central nervous system but also because of the rich pharmacology of the receptor. Many clinically important drugs including anxiolytics and anticonvulsants (benzodiazepines and barbiturates) bind to the $GABA_A$ receptor complex and modulate the behavior of its integral chloride channel [see Squires (1987)]. However, although the $GABA_A$ receptor has been well characterized with respect to its structure and ligand binding properties [reviewed in Tallman et al. (1980), Olsen (1982), Tallman and Gallager (1985), and Stephenson (1988)], much less is known about its chloride transporting properties. The purified receptor has been reconstituted into phospholipid vesicles with retention of various binding properties (Schoch et al., 1984; Sigel et al., 1985), but, to our knowledge, it has not yet been demonstrated that the reconstituted receptor retains its functional integrity. Although there have been many reports of the solubilization of the $GABA_A$ receptor complex using a variety of detergents [reviewed in Fischer and Olsen (1986)], this frequently leads to alterations in the biochemical properties of the receptor. The search for solubilization conditions that allow the receptor to retain its functional properties has therefore proved to be difficult.

Reconstitution of the $GABA_A$ receptor in a functionally active form is a prerequisite for the study of structure–function relationships and elucidation of the molecular basis for drug action. In this report, we describe the solubilization of the $GABA_A$ receptor from bovine brain with β-octyl glucoside and its reconstitution into phospholipid vesicles. The reconstituted receptor retains the ability to mediate agonist–stimulated chloride flux, and this flux response is blocked by desensitization and by GABA receptor antagonists.

Materials and Methods

Preparation of Membranes from Bovine Cerebral Cortex. Bovine brains were obtained from a local slaughterhouse. The

† This research was supported by grants from the National Science Foundation (BNS-8609693), the Epilepsy Foundation of America, and the Alcoholic Beverage Medical Research Foundation.

* Author to whom correspondence should be addressed.

‡ Present address: Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada T6G 0X9.

[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FNZ, flunitrazepam; GABA, γ-aminobutyric acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MSQ, 6-methoxy-*N*-(3-sulfopropyl)quinolinium; β-octyl glucoside, *n*-octyl β-D-glucopyranoside; PC, phosphatidylcholine; PEG, poly(ethylene glycol); PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/89/0428-2545$01.50/0

brains were immediately frozen on dry ice and stored at −80 °C until required. In each preparation, the brain was partially thawed, and approximately 100 g of cortex was removed. All operations were carried out at 0–4 °C. The cortex was chopped with a chilled razor blade and homogenized in 10 volumes of 100 mM Tris-HCl, pH 7.2, 0.3 M sucrose, 1 mM EDTA, 1 mM benzamidine, 0.5 mM DTT, 0.1 mM PMSF, 0.02 mg/mL soybean trypsin inhibitor, and 0.02% NaN_3. In the initial homogenization, a Waring blender operating at high speed was used for four 20-s periods. The mixture was then rehomogenized using a Virtis 45 at setting 30 for two periods of 20 s. The homogenate was centrifuged at 2500 rpm in a Sorvall GSA rotor for 10 min, the pellets were discarded, and the supernatant was filtered through eight layers of cheesecloth. Following centrifugation at 40 000 rpm in a Beckman type 45 Ti rotor for 45 min, the pellets were resuspended with the Virtis homogenizer in 100 mM Tris-HCl, pH 7.2, 1 mM EDTA, 1 mM benzamidine, 0.5 mM DTT, 0.01 mg/mL soybean trypsin inhibitor, 0.1 mM PMSF, and 0.02% NaN_3. The mixture was again pelleted by centrifugation for 30 min at 40 000 rpm in the type 45 Ti, and the pellets were resuspended at a concentration of 12–16 mg/mL in 100 mM Tris-HCl, pH 7.2, 1 mM EDTA, 0.5 mM DTT, 1 mM benzamidine, 0.3 mM PMSF, and 0.02% NaN_3. Protein was assayed by the method of Lowry et al. (1951), and the membranes were aliquoted in volumes of approximately 10 mL, frozen in liquid N_2, and stored at −80 °C.

Photoaffinity Labeling of the Membrane-Bound Receptor by [^{3}H]Flunitrazepam. Bovine membranes were thawed and diluted to 1 mg/mL in a final volume of 25 mL in 10 mM Hepes–Tris, pH 7.2, 150 mM NaCl, and 0.02% NaN_3. [^{3}H]FNZ (New England Nuclear) was added to a final concentration of 20 nM, and the mixture was incubated in the dark for 45 min on ice. The membranes were poured into a petri dish, and while they were continuously stirred on ice, they were irradiated at 366 nm from a distance of 5 cm for 45 min using a Spectroline EA-240 UV hand lamp. At the end of the incubation time, the membranes were collected by centrifugation for 30 min at 18 000 in a Sorvall SS34 rotor and resuspended in solubilization buffer (100 mM Tris-HCl, pH 7.2, 1 mM EDTA, 0.5 mM DTT, 1 mM benzamidine, 0.3 mM PMSF, and 0.02% NaN_3). The membranes were then subjected to cycles of centrifugation and resuspension until the radioactivity in the supernatant fell to close to the background, at which point the membranes were finally resuspended at a concentration of about 8–10 mg/mL in solubilization buffer.

SDS Gel Electrophoresis of Labeled Receptor. SDS gel electrophoresis was performed according to Laemmli (1970) by using 1-mm-thick slab gels containing 10% polyacrylamide. For determination of the labeling pattern, gel slices (0.25 cm) were cut, digested with 0.5 mL of 30% H_2O_2 for 5 h at 80 °C, and counted for ^{3}H after addition of 10 mL of 3a70 scintillation fluid (Research Products International). Molecular weight standards were obtained from Bio-Rad, and gels were stained with Coomassie Blue.

Solubilization. Membranes (approximately 8 mg of protein/mL) were stirred at 4 °C, and an equal volume of ice-cold solubilization buffer containing 2% β-octyl glucoside (Sigma Chemical Co.) and 2 mg/mL asolectin (Associated Concentrates, New York) was added dropwise. After being stirred for 30 min at 4 °C, the mixture was centrifuged for 100 min at 100000g. The supernatant was used as the solubilized receptor.

Binding of [^{3}H]Flunitrazepam to the Solubilized Receptor. A similar PEG/filtration assay to that described by Sigel et al. (1983) was used to measure the binding of [^{3}H]FNZ to the solubilized receptor; 100-μL aliquots of solubilized receptor were added to various concentrations of [^{3}H]FNZ (0–200 nM) prepared in a volume of 300 μL of 100 mM Tris-HCl, pH 7.2. Parallel control samples also included unlabeled FNZ at a final concentration of 10 μM. The samples were incubated for 120 min on ice in the dark, after which 60 μL of 22 mg/mL γ-globulin (Sigma Chemical Co.) and 340 μL of 235 mg/mL PEG (Fisher Scientific) were added to each tube. Following incubation for 30 min on ice, 0.5-mL samples were removed and filtered under vacuum through GF/C filters (Whatman) mounted in a Hoefer filtration apparatus. The filters were washed with two 5-mL volumes of 100 mM Tris-HCl, pH 7.2, containing 8% PEG, dried, and counted for ^{3}H in 5 mL of 3a70 scintillation fluid. Duplicate 80-μL samples of the incubation mixtures were also counted directly for estimates of total ligand.

Preparation of Lipids for Reconstitution. Bovine brain lipids were isolated essentially as described by Tamkun et al. (1984) for purification of rat brain lipids. Approximately 20 g of frozen bovine brain (Pel-Freeze Biologicals) was thawed and homogenized in 10 volumes of 2:1 chloroform/methanol using 12 strokes of a glass–Teflon Potter S homogenizer (B. Braun Instruments) operating at 1200 rpm. The homogenate was filtered under vacuum through a sintered glass funnel, and the filtrate was washed with 0.2 volume of 0.02% $CaCl_2$ in water. The organic phase was evaporated to dryness, taken up in a small volume of chloroform/methanol (2:1), and applied to a column (2.5 × 40 cm) of silicic acid equilibrated in chloroform. Following extensive washing with chloroform, the bound lipid was eluted with chloroform/methanol (1:1). Eluted lipids were evaporated to dryness, dissolved in 4:1 chloroform/methanol, and stored in aliquots under nitrogen at −20 °C until use. Prior to reconstitution, asolectin (Associated Concentrates, New York) dissolved in chloroform was mixed with bovine brain lipids to give final respective concentrations of 40 and 10 mg/mL. The solvent was evaporated under nitrogen at 30 °C, and the lipids were dried in vacuo for several hours. The lipid were resuspended at 75 mg/mL in solubilization buffer containing 2% β-octyl glucoside, and, after being flushed with nitrogen, the samples were vortexed vigorously to dissolve. In some experiments, approximately 0.25 μCi/mL 1,2-[^{14}C]dioleoyl-L-3-phosphatidylcholine (Amersham Corp.) was added as a radioactive lipid marker.

Reconstitution. One volume of solubilized receptor was added to 0.75 volume of resuspended lipids, and the mixture was rotated for 20 min at 4 °C. Detergent was removed by gel filtration on Sephadex G-50-80 (1.5 × 90 cm) equilibrated in 10 mM Hepes–Tris, pH 7.2, 150 mM NaCl, and 0.02% NaN_3. Reconstituted vesicles eluted in the void volume and were dialyzed for approximately 20 h against three changes of 4 L of the same buffer at 4 °C.

Sucrose Gradients. Reconstituted vesicles for sucrose gradient centrifugation were first concentrated about 10-fold by centrifugation and resuspension. Linear sucrose gradients (11.4 mL) were formed from 2% and 20% (w/v) sucrose in 10 mM Hepes–Tris, pH 7.2, 150 mM NaCl, and 0.02% NaN_3 and were overlayed on a cushion of 0.5 mL of 60% sucrose. Samples of 0.2 mL were applied to the top of the gradients which were centrifuged for 21 h at 40 000 rpm in a Beckman SW 41 rotor. Fractions of eight drops each were collected from the bottom of each tube by using a Hoefer gradient fractionator. Each fraction was counted for both ^{3}H and ^{14}C using a Beckman LS8100 scintillation counter and standard dual-isotope counting techniques.

Binding of [^{3}H]Muscimol and [^{3}H]Flunitrazepam to Reconstituted Preparations. The equilibrium binding of [^{3}H]-muscimol (New England Nuclear) and [^{3}H]FNZ to reconstituted preparations was measured by using filtration assays; 100-μL aliquots of reconstituted membranes were added to different concentrations of radiolabeled ligand to give a final volume of 800 μL and a final protein concentration of 0.1 mg/mL. Nonspecific binding was measured in the presence of 10 μM unlabeled ligand. After incubation in the dark for 60 min at 4 °C, 0.5 mL of each sample was filtered under vacuum through Whatman GF/C filters, and the filters were washed with two 4-mL volumes of cold buffer. The filters were dried, extracted overnight with 5 mL of 3a70 scintillation fluid (Research Products International), and counted for ^{3}H. Duplicate 100-μL aliquots of the incubation mixture were also removed and counted for ^{3}H in order to provide estimates of the total ligand added.

Measurement of Chloride Flux Mediated by the $GABA_A$ Receptor in Reconstituted Preparations. The fluorescence technique used to monitor chloride transport is described in detail elsewhere (Dunn et al., 1989) and is based on a technique previously developed to measure cation flux mediated by the nicotinic acetylcholine receptor in native and reconstituted vesicles (Moore & Raftery, 1980; Wu et al., 1981). Briefly, reconstituted vesicles were first washed free of chloride by centrifugation and were resuspended in 10 mM Hepes–Tris, pH 7.4, and 150 mM $NaNO_3$. Vesicles were loaded with the fluorescent probe MSQ (Molecular Probes Inc.) by two cycles of freezing and thawing in the presence of 10 mM dye. Extravesicular dye was removed by gel filtration on Sephadex G-25-300 equilibrated in the same buffer. Chloride fluxes were measured in stopped-flow experiments carried out at 25 °C. The dye-loaded vesicles were mixed with 10 mM Hepes–Tris, pH 7.4, and 150 mM NaCl in the presence and absence of drugs. Chloride influx was monitored by changes in fluorescence of the entrapped fluorophore. Flux data were analyzed by nonlinear regression techniques using the equation (Moore & Raftery, 1980):

$$F(t) = A_0 + A_1/[1 + KC_o(1 - e^{-k_1 t})] + k_0 t$$

where $F(t)$ is the fluorescence at time $= t$, A_0 is the residual fluorescence after completion of the rapid phase of quench, A_1 is the amplitude of the rapid-quench process, and k_1 is the corresponding rate constant. The term KC_o was fixed at 5.985 using the measured Stern–Volmer constant (K) of 79.8 M^{-1} (Dunn et al., 1989) and the known final concentration of chloride ions (C_o) of 75 mM. The term $k_0 t$ is used to correct for the sloping base line due to subsequent slower reactions.

Results

Photoaffinity Labeling with [^{3}H]Flunitrazepam. Previous reports of the reconstitution of the purified $GABA_A$ receptor into phospholipid vesicles have failed to demonstrate that the receptor is functionally active, i.e., retains the ability to mediate chloride flux (Schoch et al., 1984; Sigel & Barnard, 1985). At the outset of this work, we were concerned about the lability of the receptor in detergent solution [see Hammond and Martin (1986)] and the possibility that it is inactivated during prolonged purification procedures. Several detergents have been used to solubilize the $GABA_A$ receptor, but it is not known which conditions are necessary for retention of functional integrity. With these considerations in mind, it was clearly desirable to screen different solubilization conditions as rapidly as possible. We have therefore chosen to prelabel the $GABA_A$ receptor in its native membrane environment with a photoactive probe which could be used to monitor the receptor during the subsequent procedures. It has previously been shown that the benzodiazepine [^{3}H]flunitrazepam may be used as a photoaffinity label and, in the pure receptor, this ligand predominantly labels the α subunit (Sigel et al., 1983; Schoch et al., 1984; Stephenson & Barnard, 1986). The results shown in Figure 1 are in agreement with these earlier findings. Photoaffinity labeling of bovine brain membranes with [^{3}H]flunitrazepam resulted in specific covalent incorporation of label into a peptide of M_r 53 500 which is close to the apparent molecular weight reported for the α subunit (Sigel et al., 1983). The level of nonspecific labeling was very low, supporting the validity of this label as a marker for the $GABA_A$ receptor.

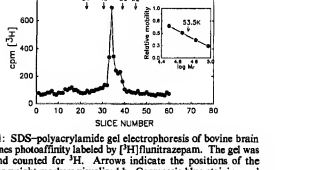

FIGURE 1: SDS–polyacrylamide gel electrophoresis of bovine brain membranes photoaffinity labeled by [^{3}H]flunitrazepam. The gel was sliced and counted for ^{3}H. Arrows indicate the positions of the molecular weight markers visualized by Coomassie blue staining and indicate that the major labeled peak runs at an apparent molecular weight of 53 500.

Solubilization of the $GABA_A$ Receptor. β-Octyl glucoside is a nonionic detergent with a high critical micelle concentration (Baron & Thompson, 1975) which makes it very attractive for reconstitution studies. This detergent has been successfully used in the solubilization and functional reconstitution of several membrane-bound receptors (see Discussion). Recently, Hammond and Martin (1986) have reported that β-octyl glucoside is also effective in solubilizing the benzodiazepine/GABA receptor complex. Preliminary studies showed that 1.5% CHAPS used previously (Sigel & Barnard, 1984) and 1% β-octyl glucoside were about equally effective in solubilizing both bovine brain protein (≃50%) and the [^{3}H]FNZ-labeled protein (≃65%). For further studies, β-octyl glucoside was therefore the detergent of choice. In data pooled from six different experiments, 71 ± 15% of the protein and 42 ± 15% of the [^{3}H]FNZ label were solubilized by this procedure.

Binding of [^{3}H]Flunitrazepam to Solubilized Receptor. In order to verify that the above estimates of the efficiency of solubilization of the $GABA_A$ receptor were not misinterpreted due to an artifact of prelabeling, the membranes were solubilized with 1% β-octyl glucoside without prior photoaffinity labeling. The results shown in Figure 2 demonstrate that the solubilized receptor binds [^{3}H]FNZ with a K_d of 1.4 nM and a B_{max} of 1.6 pmol/mg. In five different preparations of bovine brain membrane preparations, we have found that the binding of [^{3}H]FNZ is characterized by a K_d of 6.1 ± 1.5 nM and a B_{max} of 1.0 ± 0.4 pmol/mg. From these results, it may be concluded that the use of covalently labeled [^{3}H]FNZ is a valid marker for the benzodiazepine, if not the GABA, receptor.

Reconstitution of the $GABA_A$ Receptor. The conditions used for the reconstitution of the $GABA_A$ receptor were adapted from those used for the insulin receptor (Gould et al., 1982). This technique has the two major advantages of being simple and rapid, both important considerations for the reconstitution of a protein in a functional state. Chromatography

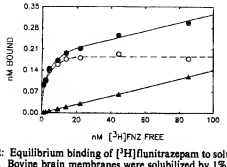

FIGURE 2: Equilibrium binding of [^{3}H]flunitrazepam to solubilized receptor. Bovine brain membranes were solubilized by 1% β-octyl glucoside, and binding was measured by filtration assays following poly(ethylene glycol) precipitation as described in the text. Total binding (●) was corrected for nonspecific binding (▲) measured in the presence of 10 μM unlabeled flunitrazepam. Specific binding (○) was fit by nonlinear regression techniques by the simple binding isotherm: bound = $R_0[L]_f/(K_d + [L]_f)$ where R_0 is the concentration of binding sites and $[L]_f$ is the concentration of free ligand. The dashed line is best fit obtained by using R_0 = 0.19 nM and K_d = 1.43 nM. Protein concentration in the assay was 0.12 mg/mL, giving a site density of 1.6 pmol/mg of protein.

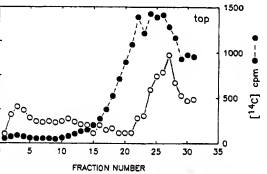

FIGURE 3: Sedimentation behavior of reconstituted vesicles on 2–20% (w/v) sucrose gradients. Vesicles that eluted in the void volume on Sephadex G-50 chromatography were concentrated about 10-fold, layered on top of linear sucrose gradients, and centrifuged for 21 h at 40 000 rpm. Fractions of approximately 0.39 mL were collected from the bottom of the tube and measured for ^{3}H and ^{14}C content.

on Sephadex G-50 was used to separate detergent and to effect reconstitution into lipid vesicles. Upon chromatography, both the $GABA_A$ receptor marker [^{3}H]FNZ and the lipid marker [^{14}C]PC eluted in the void volume which coincided with the peak of protein elution and the obvious turbity of the reconstituted membranes. Sucrose gradient centrifugation (Figure 3) has further demonstrated that the majority of the [^{3}H]-FNZ-labeled receptor comigrates with the lipid marker near the top of the sucrose gradient. In four separate experiments with each determination carried out in duplicate, it has been found that 75 ± 8% of the ^{3}H label was found in this lighter peak. This suggests that a large fraction of the receptor has indeed incorporated into lipid vesicles and that reconstitution rather than protein aggregation explains its behavior on gel filtration.

Binding of [^{3}H]Flunitrazepam and [^{3}H]Muscimol to Reconstituted Preparations. Figure 4 shows the results of an experiment in which the binding of [^{3}H]muscimol to reconstituted preparations was measured. A Scatchard plot constructed from the binding data could be described in terms of independent binding sites of low and high affinity. Fitting of this model gave estimates for the K_d's for binding to the two classes of sites of 12 nM and 0.64 μM, respectively. It should, however, be noted that this value for the low-affinity binding component is subject to large error since the maximum ligand concentration that can reasonably be used in these

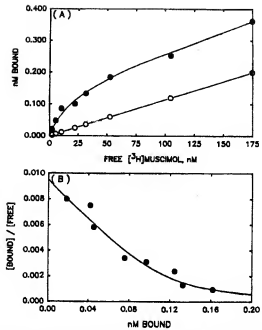

FIGURE 4: Equilibrium binding of [^{3}H]muscimol to reconstituted preparations. (A) Binding was measured in the absence (●) or presence (○) of excess unlabeled muscimol, and specific binding was calculated from the difference between the two curves. The final protein concentration in the assay was 0.1 mg/mL. (B) Scatchard plot of the data shown in (A). The solid line is the best fit by a model which assumes two classes of independent sites: $B/F = 1/2[(R_1 - B)/K_{d1} + (R_2 - B)/K_{d2} + [(R_1 - B)/K_{d1} - (R_2 - B)/K_{d2} + 4R_1K_2/K_{d1}K_{d2}]^{1/2}]$ in which B and F represent bound and free ligand, respectively, R_1 and R_2 are the concentrations of the two classes of sites, and K_{d1} and K_{d2} are the corresponding dissociation constants. Parameters obtained from curve fitting were K_{d1} = 12 nM, R_1 = 0.11 nM, K_{d2} = 0.64 μM, and R_2 = 0.296 nM.

experiments is about 200 nM and thus the low level of occupancy of the low-affinity sites precludes accurate measurement of binding site parameters. In addition, although the present data cannot rule out the presence of a homogeneous population of sites (Figure 4B), similar curvilinearity has been observed using three reconstituted preparations. Furthermore, estimates of dissociation constants are in excellent agreement with results obtained using native bovine membrane preparations (M. W. Agey and S. M. J. Dunn, unpublished observations).

The benzodiazepine [^{3}H]FNZ also bound specifically and with high affinity to reconstituted membranes. A representative experiment is illustrated in Figure 5, and a Scatchard plot gave estimates for the K_d and B_{max} of 12.4 nM and 0.491 pmol/mg, respectively. This estimate of binding affinity is higher by a factor of 2 than determinations made using native membranes (see above).

Chloride Flux Responses of the $GABA_A$ Receptor in Reconstituted Vesicles. The fluorescence method developed to measure chloride fluxes in reconstituted vesicles is described in detail elsewhere (Dunn et al., 1989). This approach has been used to verify that the $GABA_A$ receptor in reconstituted preparations retains its ability to mediate chloride transport in response to GABA receptor agonists. Vesicles were first washed to remove Cl^- ions and equilibrated in 10 mM Hepes–Tris, pH 7.4, and 150 mM $NaNO_3$. Loading of the vesicles with the chloride-sensitive fluorescent probe MSQ was achieved by cycles of freezing and thawing using liquid nitrogen (Moore & Raftery, 1980). Following the removal of extravesicular dye by gel filtration, the vesicles were rapidly mixed in a stopped-flow apparatus with buffer containing 150

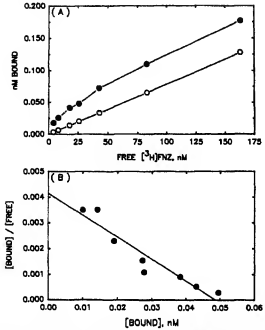

FIGURE 5: Equilibrium binding of [^{3}H]flunitrazepam to reconstituted preparations. (A) Binding was measured in the absence (●) or presence (○) of excess unlabeled flunitrazepam, allowing estimation of specific binding. (B) Scatchard plot of the data shown in (A). The solid line is a linear least-squares fit giving K_d = 12.4 nM and R_0 = 0.049 nM. Protein concentration in the assay was 0.1 mg/mL.

mM NaCl. In the absence of added ligands, some quench of fluorescence was observed (Figure 6A). This is a characteristic of the relatively high resting permeability of the membrane to chloride which allows Cl^- to enter the vesicles on fairly rapid time scales and to quench the fluorescence of the entrapped dye (Figure 6A). Inclusion of 10 μM muscimol in the chloride buffer led to a marked and rapid stimulation of chloride uptake, resulting in an increase in the magnitude of the fluorescence quench on millisecond time scales. This flux response was blocked by preincubation of the receptor with muscimol (Figure 6B), suggesting that the $GABA_A$ receptor, like other neurotransmitter receptors, desensitizes upon prolonged exposure to agonists. The muscimol-stimulated chloride uptake was also blocked by pretreatment of the vesicles with bicuculline, a competitive blocker of the $GABA_A$ receptor, or with picrotoxin, a noncompetitive antagonist. The results in Figure 6 show that although muscimol induces some stimulation of the rate of the fluorescence quench occurring on rapid time scales, the major effect is on the amplitude of this process. This is likely to be a result of the low density of $GABA_A$ receptors in these preparations which makes it unlikely that there is more than one receptor per vesicle [see Dunn et al. (1989)]. It is not possible to directly compare the magnitude of the fluorescence quench observed in different experiments. However, data obtained from experiments similar to those shown in Figure 6A using six different membrane preparations have revealed that 10 μM muscimol produces a (3.4 ± 1.4)-fold stimulation in the amplitude of the fluorescence quench occuring on rapid time scales.

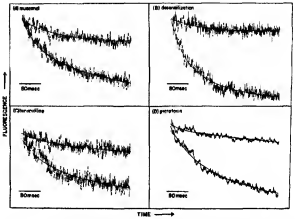

FIGURE 6: Kinetics of agonist-induced influx of Cl^- ions through reconstituted $GABA_A$ receptor channels and block by desensitization and by receptor antagonists. The data shown are representative of results obtained using at least four reconstituted preparations. For each preparation, all measurements were carried out in quadruplicate, and the traces are representative of these determinations. Data in each panel were obtained with different membrane preparations. In each experiment, MSQ-loaded vesicles equilibrated in nitrate buffer were rapidly mixed with an equal volume of buffer containing 150 mM NaCl, and the uptake of chloride was monitored by recording changes in fluorescence (measured in millivolts) of the entrapped dye. Solid lines are calculated from the best-fit parameters obtained from curve fitting as described in the text. In all traces, the contribution due to k_0 was negligible (<10 mV change in fluorescence on these time scales). (A) Effect of muscimol. Reconstituted vesicles were mixed with buffer containing NaCl in the absence (upper trace; A_1 = 58 mV, k_1 = 0.99 s^{-1}) or presence (lower trace; A_1 = 152 mV, k_1 = 2.2 s^{-1}) of 10 μM muscimol. (B) Inhibition of chloride influx by desensitization. In the upper trace, membranes were incubated with 10 μM muscimol for 10 min prior to mixing with 10 μM muscimol in chloride buffer (A_1 = 67 mV, k_1 = 0.71 s^{-1}). The lower trace is the control in which the membranes were not preincubated with muscimol (A_1 = 198 mV, k_1 = 1.62 s^{-1}). (C) Effect of bicuculline. In the upper trace, membranes were incubated with 50 μM bicuculline before mixing with 50 μM bicuculline and 10 μM (final concentration) muscimol (A_1 = 39 mV, k_1 = 0.89 s^{-1}). The lower trace was recorded in the absence of bicuculline (A_1 = 173 mV, k_1 = 1.1 s^{-1}). (D) Effect of picrotoxin. Same experiment as in (C) except that in the upper trace 100 μM picrotoxin was used in the preincubation and the buffer used for mixing (A_1 = 12 mV, k_1 = 0.77 s^{-1}). The lower trace is the control in the absence of picrotoxin (A_1 = 114 mV, k_1 = 1.7 s^{-1}). In this experiment, the traces shown are signal averaged (traces/average = 4), and this accounts for the lower noise level compared to panels A–C. However, the data shown are again representative of four such averages.

Discussion

Despite extensive studies of $GABA_A$ receptor solubilization [reviewed by Fischer and Olsen (1986)], there have been no previous reports concerning the functional integrity of the solubilized receptor preparations. The major finding reported here is that the $GABA_A$ receptor may be solubilized from bovine brain membranes using the nonionic detergent β-octyl glucoside and may be reconstituted into lipid vesicles in a functionally active form. Chloride uptake by reconstituted preparations was markedly stimulated by the GABA receptor agonist muscimol and was blocked by desensitization and by the receptor antagonists bicuculline and picrotoxin. In addition, the properties of binding of [^{3}H]muscimol and the benzodiazepine [^{3}H]flunitrazepam to the reconstituted preparations were very similar to those measured for the receptor in its native membrane-bound state. This demonstration of functional reconstitution represents an important step in the study of structure–function relationships of the GABA receptor and its integral chloride ion channel.

In preliminary experiments, the membrane-bound receptor was first covalently labeled with the photoactive ligand [^{3}H]FNZ which was used as a marker in the development of a successful reconstitution protocol. The label was specifically incorporated into a peptide whose molecular weight corresponds to that previously reported for the α subunit (Sigel et

al., 1983; Schoch et al., 1984). More recently, the β subunit has been implicated as being involved in the binding of GABA analogues since this subunit was photoaffinity labeled by [^{3}H]muscimol (Casalotti et al., 1986). It has not, however, been clearly established that benzodiazepines bind exclusively to the α subunit since in several studies it has been found that upon irradiation with UV light, [^{3}H]FNZ irreversibly binds to several proteins with apparent molecular weights between 51 000 and 62 000 [see Fuchs et al. (1988)]. In addition, the α subunit of the purified $GABA_A$ from rat brain has recently been reported to be heterogeneous, and three different proteins of molecular weights 51 000, 53 000, and 59 000 which were recognized by an α-subunit-specific antibody were also photoaffinity labeled by [^{3}H]FNZ (Fuchs et al., 1988).

The $GABA_A$ receptor has previously been solubilized using the zwitterionic detergent CHAPS (Stephenson & Olsen, 1982; Mernoff et al., 1983; Sigel & Barnard, 1984). In this medium, the solubilized protein exhibits many of the characteristics of the receptor in its native membrane state, including barbiturate stimulation of [^{3}H]FNZ binding and high-affinity binding sites for the channel-blocking ligand *tert*-butyl[^{35}S]bicyclophosphorothionate (TBPS; Sigel & Barnard, 1984). Recently, Hammond and Martin (1986) compared the effectiveness of CHAPS and β-octyl glucoside in solubilizing the GABA/benzodiazepine receptor from rat cerebellar membranes and concluded that β-octyl glucoside offers some advantages for solubilization and reconstitution studies. In particular, reconstitution of the β-octyl glucoside solubilized receptor into liposomes seemed to improve the stability of receptor binding properties, a property that was not shared by the CHAPS-solubilized receptor. Another compelling reason for selecting β-octyl glucoside in the present study was that this detergent has previously been used successfully in the solubilization and reconstitution of other membrane-bound receptors, including the insulin receptor (Gould et al., 1982), the nicotinic acetylcholine receptor (Paraschos et al., 1982), and the β-adrenergic receptor (Cerione et al., 1983). Bovine membranes were, therefore, solubilized by 1% β-octyl glucoside in the presence of 1 mg/mL asolectin, the latter being added to stabilize the receptor during solubilization (Bristow & Martin, 1987).

Reconstitution of the $GABA_A$ receptor in a functional state appears to be dependent on the lipid composition of the reconstituted membrane. Asolectin alone was not sufficient to stabilize the functional properties of the $GABA_A$ receptor (data not shown). The receptor has therefore been reconstituted using a 4:1 mixture of asolectin/bovine brain lipids. Since the composition of the endogenous lipid preparations has not been analyzed in detail, the specific lipid requirement for successful reconstitution is not yet known. It is, however, interesting to note that in a previous study of the binding properties of the CHAPS-solubilized receptor from rat cerebellar membranes it was shown that binding was protected by the presence of a natural brain lipid extract and cholesteryl hemisuccinate but not by asolectin (Bristow & Martin, 1987).

Confirmation that the $GABA_A$ receptor was successfully reconstituted into lipid vesicles during gel filtration has come from the comigration of the [3H]FNZ receptor marker and a [^{14}C]PC lipid marker on both the gel filtration column and during centrifugation on 2–20% sucrose gradients. In five experiments, the labeled receptor failed to migrate far into the sucrose gradient, suggesting that most of the protein is incorporated into lipid vesicles and that there is little contribution from protein that has aggregated during detergent removal since this would have migrated to the bottom of the tube. The reconstituted receptor retained the ability to bind both [^{3}H]muscimol and [^{3}H]FNZ, and the estimated binding affinities for these ligands were in good agreement with those measured for binding to native membrane preparations. Furthermore, the binding of [^{3}H]muscimol appears to involve both high- and low-affinity sites which may have important implications for the function of the $GABA_A$ receptor.

The reconstituted $GABA_A$ receptor mediates a rapid influx of Cl^- in response to the binding of muscimol. Preincubation of the membrane preparations with muscimol abolished this response, suggesting that prolonged exposure of the receptor to the agonist results in desensitization. In several, but not all, previous studies the $GABA_A$ receptor has been shown to desensitize [reviewed by Cash and Subbarao (1987); see also Dunn et al. (1989)]. Chloride influx was also blocked by the receptor antagonist bicuculline and by the putative channel blocker picrotoxin, demonstrating the specificity of the response for the $GABA_A$ receptor.

In conclusion, we describe here the functional reconstitution of the $GABA_A$ receptor after solubilization from bovine brain membranes. This is a prerequisite for future studies of the structural requirements for the ligand-gated chloride channel of the receptor protein and provides a new approach for studying the mechanisms of drug action in this pharmacologically rich central nervous system receptor.

Acknowledgments

We are very grateful to Dr. Michael Raftery for generously lending us his stopped-flow instrumentation for use in these studies. We also thank Dr. Arthur Spector for his advice on reconstitution techniques, Rick Shelman for preliminary studies, and Tom Lanigan and Tuan Bui for expert technical assistance.

Registry No. Cl^-, 16887-00-6; β-octyl glucoside, 29836-26-8.

References

Baron, C., & Thompson, T. E. (1975) *Biochim. Biophys. Acta 382*, 276–285.

Bristow, D. R., & Martin, I. L. (1987) *J. Neurochem. 49*, 1386–1393.

Casalotti, S. O., Stephenson, F. A., & Barnard, E. A. (1986) *J. Biol. Chem. 261*, 15013–15016.

Cash, D. J., & Subbarao, K. (1987) *Biochemistry 26*, 7556–7562.

Cerione, R. A., Strulovici, B., Benovic, J. L., Strader, C. D., Caron, M. C., & Lefkowitz, R. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4899–4903.

Dunn, S. M. J., Shelman, R. A., & Agey, M. W. (1989) *Biochemistry* (following paper in this issue).

Fischer, J. B., & Olsen, R. W. (1986) in *Benzodiazepine/GABA Receptors and Chloride Channels: Structural and Functional Properties* (Olsen, R. W., & Venter, J. C., Eds.) pp 241–259, Alan R. Liss, New York.

Fuchs, K., Möhler, H., & Siegert, W. (1988) *Neurosci. Lett. 90*, 314–319.

Gould, R. J., Ginsberg, B. H., & Spector, A. A. (1982) *J. Biol. Chem. 257*, 477–484.

Hammond, J. R., & Martin, I. L. (1986) *J. Neurochem. 47*, 1161–1171.

Kuriyama, K., & Taguchi, J. (1987) *Neurochem. Int. 10*, 253–263.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lowry, O. H., Rosenbrough, N. J., Farr, A., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Mernoff, S. T., Cherwinski, H. M., Becker, J. W., & de Blas, A. L. (1983) *J. Neurochem. 41*, 752–758.

Moore, H.-P. H., & Raftery, M. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4509–4513.
Olsen, R. W. (1982) *Annu. Rev. Pharmacol. Toxicol. 22*, 245–277.
Paraschos, A., Gonzalez-Ros, J. M., & Martinez-Carrion, M. (1982) *Biochim. Biophys. Acta 691*, 249–260.
Schoch, P., Häring, P., Takas, B., Stähli, C., & Möhler, H. (1984) *J. Recept. Res. 4*, 189–200.
Schofield, P. R., Darlison, M. J., Fujita, N., Burt, D. R., Stephenson, F. A., Rodriguez, H., Rhee, L. M., Ramachandran, J., Reale, V., Glencorse, T. A., Seeburg, P. H., & Barnard, E. A. (1987) *Nature (London) 328*, 221–227.
Sigel, E., & Barnard, E. A. (1984) *J. Biol. Chem. 259*, 7219–7223.
Sigel, E., Stephenson, F. A., Mamalaki, C., & Barnard, E. A. (1983) *J. Biol. Chem. 258*, 6965–6971.
Sigel, E., Mamalaki, C., & Barnard, E. A. (1985) *Neurosci. Lett. 61*, 165–170.
Squires, R. F., Ed. (1987) *GABA and Benzodiazepine Receptors*, Vol. I and II, CRC Press, Inc., Boca Raton, FL.
Stephenson, F. A. (1988) *Biochem. J. 249*, 21–32.
Stephenson, F. A., & Olsen, R. W. (1982) *J. Neurochem. 39*, 1579–1586.
Stephenson, F. A., & Barnard, E. A. (1986) in *Benzodiazepine/GABA Receptors and Chloride Channels: Structural and Functional Properties* (Olsen, R. W., & Venter, J. C., Eds.) pp 261–274, Alan R. Liss, New York.
Tallman, J. F., & Gallager, (1985) *Annu. Rev. Neurosci. 8*, 21–44.
Tallman, J. F., Paul, S. M., Skolnick, P., & Gallager, D. W. (1980) *Science (Washington, D.C.) 207*, 274–281.
Tamkun, M. M., & Catterall, W. A. (1984) *J. Biol. Chem. 256*, 11457–11463.
Wu, W. C.-S., Moore, H.-P. H., & Raftery, M. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 775–779.

Fluorescence Measurements of Anion Transport by the $GABA_A$ Receptor in Reconstituted Membrane Preparations†

Susan M. J. Dunn,*,‡ Rick A. Shelman, and Michael W. Agey
Department of Physiology and Biophysics, The University of Iowa, Iowa City, Iowa 52242
Received August 9, 1988; Revised Manuscript Received October 27, 1988

ABSTRACT: A fluorescence assay for measuring the functional properties of the $GABA_A$ receptor in reconstituted membrane vesicles is described. This assay is based on a method previously described to measure monovalent cation transport mediated by the nicotinic acetylcholine receptor in membranes from *Torpedo* electric organ [Moore, H.-P. H., & Raftery, M. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4509–4513]. The $GABA_A$ receptor has been solubilized from bovine brain membranes and reconstituted into phospholipid vesicles. Influx of chloride or iodide into the vesicles has been measured in stopped-flow experiments by monitoring the fluorescence quench of an anion-sensitive fluorophore trapped within the vesicles. Muscimol, a $GABA_A$ receptor agonist, stimulated a rapid uptake of either chloride or iodide. Stimulation of chloride influx was dependent on the concentration of muscimol, and the midpoint of the dose–response curve occurred at approximately 0.3 μM. Agonist-stimulated uptake was enhanced by diazepam and blocked by desensitization and by the antagonists bicuculline and picrotoxin. These receptor-mediated effects are shown to be qualitatively similar to measurements of $^{36}Cl^-$ and $^{125}I^-$ efflux using synaptoneurosomes prepared from rat cerebral cortex. The advantages of the fluorescence method in terms of its improved time resolution, sensitivity, and suitability for quantitating $GABA_A$ receptor function are discussed.

The interaction of γ-aminobutyric acid (GABA)[1] with its receptor results in the opening of a chloride ion channel which appears to be an integral part of the receptor–protein complex (Schofield et al., 1987). GABA is the major inhibitory neurotransmitter in mammalian brain, and modulation of GABAergic transmission has a profound effect on cellular excitability. Many pharmacological agents act on the $GABA_A$ receptor and alter its chloride-transporting properties. In addition to binding sites for GABA and its analogues, the receptor also has distinct sites for benzodiazepines, for barbiturates, and for the alkaloid picrotoxin (Olsen, 1982). Both benzodiazepines and barbiturates potentiate the actions of GABA but apparently by different mechanisms. Benzodiazepines have been shown to increase the affinity of the receptor for GABA (Tallman et al., 1978; Wastek et al., 1978; Briley & Langer, 1978; Martin & Candy, 1978; Karobath & Sperk, 1979) whereas barbiturates appear to prolong channel opening time [reviewed by Olsen (1981) and Tallman and Gallager (1985)].

Although electrophysiological experiments suggested that the interaction of GABA with its receptor results in an increase in chloride ion conductance (Krnjevic, 1974; McBurney & Barker, 1978; Nistri et al., 1980), only recently has this been demonstrated to occur in cell-free membrane preparations from brain. Synaptoneurosomes have been used to measure $^{36}Cl^-$ uptake or release in response to added GABA (Harris & Allan,

† This work was supported by NSF Grant BNS-8609693, by NIH Grant DK 25295, and by grants from the Epilepsy Foundation of America and the Alcoholic Beverage Medical Research Foundation.
* Author to whom correspondence should be addressed.
‡ Present address: Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada T6G 0X9.

[1] Abbreviations: GABA, γ-aminobutyric acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MSO, 6-methoxy-*N*-(3-sulfopropyl)quinolinium; β-octyl glucoside, *n*-octyl β-D-glucopyranoside; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/89/0428-2551$01.50/0